THE STRUCTURES AND FUNCTIONS OF OLIGOSACCHARINS

December 15, 1989-December 14, 1992

Year 2 Continuation

Progress Report

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Introduction.

Abstracts of papers published or in press reporting work supported by this grant proposal during the past year are given here to describe the progress we have made in this research project.

Cervone, F., G. De Lorenzo, R. D'Ovidio, M.G. Hahn, Y. Ito, A. Darvill, and P. Albersheim. 1989. Phytotoxic effects and phytoalexin-elicitor activity of microbial pectic enzymes. In: *Phytotoxins & Plant Pathogenesis* (A. Graniti et al., eds.) NATO ASI Series Vol. H27. Springer-Verlag, Berlin-Heidelberg, pp. 473-477.

Microbial pectic enzymes not only macerate plant tissue but also kill plant cells and elicit synthesis and accumulation of phytoalexins. We have evidence suggesting that these three physiological activities of pectic enzymes may be regulated by factors such as endo-polygalacturonase inhibiting proteins (PGIP) and pH.

Eberhard, S., N. Doubrava, V. Marfà, D. Mohnen, A. Southwick, A. Darvill, and P. Albersheim. 1989. Pectic cell wall fragments regulate tobacco thin-cell-layer morphogenesis. *The Plant Cell* 1:747-755.

Pectic fragments of cell wall polysaccharides, released from the walls of suspension-cultured sycamore cells by treatment with endopolygalacturonase, were tested for morphogenesis-regulating activity in a modified tobacco thin-cell-layer explant (TCL) bioassay. The pectic fragments inhibited the formation of roots on TCLs grown on a root-inducing medium containing 15 micromolar indole-3-butyric acid and 0.5 micromolar kinetin. Addition of the pectic fragments to a root-inducing medium containing 7 micromolar indole-3-butyric acid and 0.15 micromolar kinetin caused roots to form on the basal end of TCLs. TCLs cultured on this medium in the absence of added pectic fragments formed roots along their entire length. The pectic fragments induced polar tissue enlargement and the formation of flowers on TCLs cultured on transition medium. The flower-inducing activity was stable to heat treatment and proteolytic digestion. Pectic fragments isolated from the walls of suspension-cultured tobacco cells were as effective as those from the walls of sycamore cells in inducing *de novo* flower formation in the TCLs. These results support the hypothesis that oligosaccharins from plant cell walls regulate morphogenesis.

Hahn, M.G., P. Bucheli, F. Cervone, S.H. Doares, R.A. O'Neill, A. Darvill, and P. Albersheim. 1989. Roles of cell wall constituents in plant-pathogen interactions. In: *Plant-Microbe Interactions*, Vol. 3 (E. Nester and T. Kosuge, eds.) McGraw-Hill, pp. 131-181.

Pathogens find themselves most commonly in the presence of plants other than their hosts. Under such circumstances, a pathogen fails in its efforts to initiate infection. What is it that renders a plant's environment inhospitable to a pathogen except in the rare instance when the plant happens to be a susceptible host? It is this question that we propose to answer by our hypothesis that, in many instances of pathogenesis by bacteria or fungi, it is an interaction between the pathogen and the carbohydrates of the host that determines the pathogen's ability to produce enzymes capable of degrading the host's cell walls.

Doares, S.H., P. Bucheli, P. Albersheim, and A.G. Darvill. 1989. Host-Pathogen Interactions XXXIV. A heat-labile activity secreted by a fungal phytopathogen releases fragments of plant cell walls that kill plant cells. *Mol. Plant-Microbe Interac.* 2:346-353.

Magnaporthe grisea (anamorphs: *Pyricularia oryzae* Cav., *P. grisea*), the ascomycetous causal agent of rice blast, was grown in liquid shake culture either on a minimal salts medium with a selected carbon source or on a complete (yeast extract-casein hydrolysate-sucrose) medium. When grown on pectin or isolated plant cell walls as the carbon source, the fungus secreted, into the culture medium, a heat-labile activity capable of killing plant cells. Plant cell death was monitored by the ability of suspension-cultured maize cells to incorporate [^{14}C]leucine into acid-precipitable material. The heat-labile killing activity was neither secreted when the fungus was grown on sucrose or xylan nor produced when the fungus was grown on a complete medium. Heat-stable wall fragments, capable of killing plant cells, were released from isolated maize suspension-cultured cell walls treated with the heat-labile killing activity.

Doares, S.H., P. Bucheli, P. Albersheim, and A.G. Darvill. 1989. Fungal enzymes, plant cell wall fragments, and plant cell death -- a possible mechanism for hypersensitivity in the rice blast system. In: *Signal Molecules in Plants and Plant-Microbe Interactions* (B.J.J. Lugtenberg, ed.), NATO ASI Series, Vol H36, Springer-Verlag, Berlin-Heidelberg, pp. 229-233.

The hypersensitive response is a commonly observed phenomenon in plant pathology. A plant, when challenged by a microbe that is either a species that is not a pathogen of that plant or an incompatible race of a species that is a pathogen of other cultivars of the plant, commonly exhibits a hypersensitive resistance response, which is phenotypically observed as the rapid necrosis of one or a few plant cells at the site of attempted infection. This necrosis is presumed to result in processes that limit the spread of the pathogen.

The biochemical mechanism by which hypersensitive cell death is triggered has not been established. Various molecules or preparations have been suggested as inducers of the hypersensitive response; these include cell wall preparations from *Phytophthora infestans* that kill potato tuber tissue, lipids from *P. infestans* that kill potato tissue, a small peptide from intercellular fluids of fungal-infected tomato that kills tomato tissue, and a glycan that causes cell

death in wheat leaves. The glycoprotein from which the glycan was obtained was derived from the germ tube walls of the uredospores of the fungal pathogen *Puccinia graminis* f.sp. *tritici*. It should be noted that the origin of all of these suggested inducers of hypersensitivity (with the possible exception of the small peptide from fungal-infected tomato tissue) is the pathogen rather than the host.

Various physiological responses in the plant have been described that are associated with the hypersensitive response, including activation of the plasmalemma K^+/H^+ exchange, increased consumption of ATP, increased protein synthesis, production of superoxide anion, increased lipoxygenase activity, an increase in lignin deposition, increased cell wall hydroxyproline content, and a decrease in the components that were able to kill the cells of potato tuber, cucumber fruit, or tobacco pith tissues when isolated tobacco pith tissue cell walls were treated with a pectate lyase from *Erwinia*. We were also unable to obtain wall fragments with killing activity by treating isolated maize cell walls with the pectin lyase secreted by *M. grisea*. Our results also do not confirm those of Ishii, who reported that the filtrates of rice cells treated with a commercially available xylanase did not contain heat-stable components that kill rice cells, even though Ishii reported that the xylanase itself was able to kill rice cells.

Cervone, F., G. De Lorenzo, G. Salvi, C. Bergmann, M.G. Hahn, Y. Ito, A. Darvill, and P. Albersheim. 1989. Release of phytoalexin elicitor-active oligogalacturonides by microbial pectic enzymes. In: *Signal Molecules in Plants and Plant-Microbe Interactions* (B.J.J. Lugtenberg, ed.), NATO ASI Series, Vol H36, Springer-Verlag, Berlin-Heidelberg, pp. 85-89.

Linear α -1,4-linked oligogalacturonides of chain lengths between 10 and 13 have been shown to elicit phytoalexin accumulation and lignification in plants, but shorter oligogalacturonides do not have this effect. Phytoalexin elicitor-active oligogalacturonides are thought to be released from the plant cell wall by the action of two groups of microbial pectic-degrading enzymes, the endopolygalacturonases and the endopectate lyases. These enzymes release elicitor-active oligogalacturonides from polygalacturonic acid *in vitro* but also quickly depolymerize the elicitor-active chains, converting them into shorter, elicitor-inactive molecules. We will discuss two mechanisms by which the action of fungal endopolygalacturonases and bacterial endopectate lyases may be regulated such that the formation of elicitor-active oligogalacturonides is favored.

Darvill, A.G., P. Albersheim, P. Bucheli, S. Doares, N. Doubrava, S. Eberhard, D.J. Gollin, M.G. Hahn, V. Marfà-Riera, W.S. York, and D. Mohnen. 1989. Oligosaccharins -- plant regulatory molecules. In: *Signal Molecules in Plants and Plant-Microbe Interactions* (B.J.J. Lugtenberg, ed.), NATO ASI Series, Vol H36, Springer-Verlag, Berlin-Heidelberg, pp. 41-48.

The discoveries that complex carbohydrates are tissue-specific cell-surface antigens and the receptors for hormones, toxins, bacteria, and viruses have created considerable interest in complex carbohydrates. Furthermore, the complex carbohydrate portions of some glycoprotein hormones are required for their biological activity, and the complex carbohydrates of some glycoprotein enzymes keep the enzymes stable, while the carbohydrates of some glycoproteins direct the glycoproteins to their proper locations. Moreover, the immune

response to glycoproteins is often directed to their carbohydrate side chains, and the carbohydrate chains have been shown to affect the activity and residence time of glycoprotein pharmaceuticals. Still another discovery that has generated considerable interest in complex carbohydrate research is that these molecules perform regulatory functions in plants and animals, often at the level of controlling gene expression.

Our laboratory has long pursued two seemingly unrelated lines of research -- one, elucidating the structure and function of the matrix polysaccharides of the walls of growing plant cells, and the other, studying how plants defend themselves against disease. The combined results from these projects led to our discovery that structurally defined fragments of plant cell wall polysaccharides are able to function in plants as chemical messages with specific regulatory properties. We call oligosaccharides with regulatory activity *oligosaccharins*. We have determined that oligosaccharins not only trigger plant defenses against pathogens and other types of stress, but also are able to regulate the rate of growth and differentiation of plant tissues into roots, flowers, and vegetative buds.

The first characterized oligosaccharins were those that are commonly referred to as elicitors of defense responses in plants. The best oligogalacturonides are effective inhibitors of root formation and the oligogalacturonides can control the location on the explants where roots form. Thus, it is becoming apparent that oligogalacturonides of specific size are extremely important regulators of plant growth and development; oligogalacturonides may have as many functions as any of the classical phytohormones. Indeed, we believe that oligogalacturonides may be as important and as effective in the control of plant growth and development as auxin, cytokinin, gibberellin, abscisic acid, or ethylene. As with the phytohormones, it remains to be determined how oligogalacturonides control growth and development. Nevertheless, oligogalacturonides are very important regulatory molecules of plants.

Mohnen, D., S. Eberhard, V. Marfà, N. Doubrava, P. Toubart, D.J. Gollin, T. Gruber, W. Nuri, P. Albersheim, and A. Darvill. 1990. The control of root, vegetative shoot and flower morphogenesis in tobacco thin cell-layer explants (TCLs). *Development* 108:191-201.

Thin cell-layer explants (TCLs) have been proposed as favorable tissues for the study of root, vegetative shoot and flower formation. We tested the effects of pH, light quality, light quantity, and IBA and kinetin concentrations on the morphogenesis of TCLs cultured individually on a liquid medium. Alterations of the amounts of exogenously supplied IBA and kinetin were sufficient to induce the formation of roots, vegetative shoots and flowers on TCLs cultured on otherwise identical media. The type and number of organs formed were sensitive to the intensity of light (55, 75, 100 and 120 $\mu\text{Einsteins m}^{-2}\text{sec}^{-1}$) under which TCLs were grown. Evidence was obtained that the effects of light on TCL morphogenesis were associated with photochemical degradation of IBA in the medium. Evaluation of the organogenesis that occurred in TCLs cultured on a medium containing a range of IBA and kinetin concentrations showed that the number and type of organs formed, and overall growth, were dependent upon the initial concentrations of auxin and cytokinin. We have developed the TCL culture system into a sensitive and reproducible bioassay for the study of morphogenesis. The advantages of using the TCL morphogenesis bioassay for the identification and study of molecules (e.g., cell wall oligosaccharides) that may regulate morphogenesis are discussed.

Bucheli, P., S.H. Doares, P. Albersheim, and A. Darvill. 1990. Host-pathogen interactions XXXVI. Partial purification and characterization of heat-labile molecules secreted by the rice blast pathogen that solubilize plant cell wall fragments that kill plant cells. *Physiol. and Mol. Plant Pathol.* 36:159-173.

Heat-labile factors capable of killing plant cells are secreted by the rice pathogen *Magnaporthe grisea* when grown on rice cell walls. Inhibition of [14 C]-leucine incorporation into maize cells (*Zea mays* cv. Black Mexican Sweet) was shown to be as reliable as the vital dyes 2,3,5-triphenyltetrazolium chloride and fluorescein diacetate for assessing cell viability. The heat-labile factors responsible for killing plant cells were partially purified by CM-Sephadex and Superose 12 chromatography. A combination of four of the Superose 12 column fractions synergistically killed the plant cells; the killing activity of the combined fractions was 2.5 times as high as that obtained by the sum of the four fractions assayed individually. We purified to apparent homogeneity pectin lyase (PL), pectin methylesterase (PME), and xylanase from the fungal culture filtrate. When these enzymes were tested in various combinations and at the same concentrations as they were found in the culture filtrate, they did not kill plant cells. The same enzymes were not able to release fragments that killed plant cells from isolated maize cell walls, whereas fractions containing the partially purified heat-labile killing activity rapidly released heat-stable maize cell wall fragments that killed maize cells. The results of this study indicate that a heat-labile killing activity secreted by *M. grisea*, which probably consists of two or more factors (presumably proteins), solubilizes from maize cell walls heat-stable fragments (presumably carbohydrates) that kill maize cells. Furthermore, although pectic enzymes may prove to be necessary for killing, the pectic enzymes in the culture filtrate of *M. grisea* do not, by themselves, kill maize cells.

Cervone, F., G. De Lorenzo, R. Pressy, A.G. Darvill, and P. Albersheim. 1990. Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? *Phytochemistry* 29:447-449.

The effect of a polygalacturonase-inhibiting protein (PGIP) isolated from *Phaseolus vulgaris* on the activities of pectic enzymes of microbial and plant origin was investigated. The PGIP inhibited all fungal endopolygalacturonases tested, but did not inhibit the activity of a bacterial endopolygalacturonase and a bacterial endopectate lyase. The protein was also unable to inhibit tomato endopolygalacturonase and exopolygalacturonases from water oak and sorghum pollen. Surprisingly, the protein inhibited exopolygalacturonase from corn pollen.

De Lorenzo, G., Y. Ito, R. D'Ovidio, F. Cervone, P. Albersheim, and A.G. Darvill. 1990. Host-pathogen interactions XXXVII. Abilities of the polygalacturonase-inhibiting proteins from four cultivars of *Phaseolus vulgaris* to inhibit the endopolygalacturonases from three races of *Colletotrichum lindemuthianum*. *Physiol. Mol. Plant Pathol.* 36:421-435.

The ability of endopolygalacturonases isolated from three races of *Colletotrichum lindemuthianum* to form phytoalexin elicitor-active oligogalacturonides was studied in the absence and presence of endopolygalacturonase-inhibiting proteins (PGIPs) isolated from four varieties of *Phaseolus vulgaris*. Endopolygalacturonases (each 40 kDa on SDS-PAGE) from the α , β and γ races of *Colletotrichum lindemuthianum* were purified by ion-

exchange and fast-protein-liquid gel-permeation chromatographies. The polygalacturonases from the three races formed elicitor-active oligogalacturonides at the same rate. PGIPs (each 41 kDa) were purified from four different cultivars of *Phaseolus vulgaris* (cvs Great Northern, Pinto, Red Kidney, and Small Red) by affinity chromatography through a Sepharose-endopolygalacturonase column. The PGIPs from all four cvs of *P. vulgaris* were able to increase the lifetimes of phytoalexin elicitor-active oligogalacturonides generated by the endopolygalacturonases. We observed no significant differences in the rate and pattern of formation of elicitor-active oligogalacturonides by the three enzymes in the presence of each of the four PGIPs. Our data indicate that the interaction between endopolygalacturonases and their inhibitor proteins does not *per se* explain race-cultivar specificity.

Hahn, M.G., A. Darvill, P. Albersheim, C. Bergmann, J.-J. Cheong, A. Koller, and V.-M. L. 1990. Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation. In: *Molecular Plant Pathology: A Practical Approach* (D.J. Bowles, ed.) IRL Press. In press.

Plants utilize a variety of defense responses when confronted with invasive microorganisms. These defense responses include such diverse mechanisms as the synthesis and accumulation of low molecular weight anti-microbial compounds called phytoalexins, the production of glycosyl hydrolases capable of attacking microbial surface polymers, the synthesis of proteins that inhibit microbial degradative enzymes, and the modification of plant cell walls by deposition of callose, hydroxyproline-rich glycoproteins, and/or lignin.

Biochemical analysis of the induction of plant defense responses has been facilitated by the recognition that cell-free extracts of microbial and plant origin are capable of inducing defense responses when applied to plant tissues. The active components in the extracts are commonly referred to as "elicitors". The term "elicitor" was originally used to refer to molecules and other stimuli that induce phytoalexin synthesis in plant cells, but the term is now used to describe molecules that elicit any of the observed plant defense responses.

Crude preparations of biotic elicitors have been prepared from a variety of sources. Elicitors that have been purified to apparent homogeneity include oligosaccharide fragments of fungal and plant cell wall polysaccharides, and fungal cell surface lipids. A number of elicitor-active polypeptides of fungal and bacterial origin have also been purified and partially characterized. Several of these have been shown to be glycosylhydrolases that release elicitor-active oligosaccharides from plant cell walls. Other elicitor-active polypeptides have not (yet) been shown to have enzymatic activities. Oligosaccharide elicitors that derive from plant and fungal cell wall polysaccharides are the best-characterized elicitors. Their purification and characterization will be described in detail in this chapter.

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